

APPLICANTS: Chagnaud *et al.*
U.S.S.N.: 09/331,980

#11/B

AMENDMENTS UNDER 37 C.F.R. §1.111JM
12/3/01***In the Specification:***

On page 1, line 4, please insert the following sentence:

-- CLAIM OF PRIORITY

This application is a 35 U.S.C. 371 national application of PCT/FR97/02412, filed on December 23, 1997. This application also claims priority to French Patent Application FR 96/16207.

TECHNICAL FIELD OF THE INVENTION--

On pg. 1, line 14, insert **--BACKGROUND OF THE INVENTION--**.

Please replace the paragraph beginning on page 1, line 15, with the following rewritten paragraph:

B Nitric oxide, hereinafter designated also as NO, is described as being the smallest molecule made by the cells. Initially assimilated to endothelium derived relaxing factor (EDRF), it was then recognized as a neuromediator, and is thought to be the first neurotransmitter with retrograde activity, as well as a cytostatic/cytotoxic molecule. Because of its strong reactivity, nitric oxide is capable of reacting with a large number of molecules to form conjugates which have multiple functions and therefore participate in many physiological and pathophysiological processes.

On page 2, line 27, insert **--SUMMARY OF THE INVENTION--**.

B On page 2, line 28, insert **--DESCRIPTION OF THE DRAWINGS**

Fig. 1 is a graph illustrating the zone of absorbency of NO-Tyr-BSA;

Fig 2 is a graph illustrating the zone of absorbency of NO-Cys-BSA;

Fig. 3 is a graph illustrating response following immunization with NO-Tyr-BSA;

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Fig. 4 is a graph illustrating response following immunization with NO-Cys-BSA;
Fig. 5 is a graph illustrating the avidity of conjugated anti-NO-Tyr Ab and
conjugated anti-NO=Cys in competition tests;

Fig. 6 illustrates the kinetics of the formation and concentration in NO-Cys-BSA
formed in supernatant of the culture of activated macrophages determined at incubation times: 0,
3, 4, 6, 8, 11, 14, 18 and 20 hours using "C" antiserum;

Fig. 7 illustrates inhibition of the cytostatic effect of the BCG macrophages on the
T. musculi *in vitro* in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100;

Fig. 8 illustrates the cytostatic effect of supernatants containing NO-BSA from
activated macrophages added to normal macrophages containing *T. musculi*. Inhibition of this
effect in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100;

Fig. 9 illustrates the spectrometric analysis of the NO-Cys-G-BSA immunogen
and its structural homologue Cys-G-BSA based on wavelength;

Fig. 10 illustrates the evolution of the antibody response during immunization of
the intraperitoneally immunized mouse;

Fig. 11 illustrates the evolution of the antibody response during immunization of
the intraperitoneally immunized mouse;

Fig. 12 illustrates the avidity and specificity of the anti-NO-Cys-G antibodies in
the mouse (IP);

Fig. 13 illustrates represents the avidity and specificity of the anti-NO-Cys-G
mouse antibodies (PC);

Fig. 14 illustrates the avidity and specificity of the anti-NO-Cys-G monoclonal
Ab;

Fig. 15a is a high-magnification (100X) immunocytochemical marking illustrating
anti-NO-Cys-G monoclonal antibody and showing very clear markings (immunoreactivities) in
terms of trypanosomes co-cultivated in the presence of the activated macrophages;

Fig. 15b is a high-magnification (100X) immunocytochemical marking illustrating
a much weaker marking obtained in the co-culture of activated macrophages / trypanosomes, in
the presence of NMMA (0.5 mM);

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Fig. 15c is a high-magnification (100X) immunocytochemical marking illustrating a total absence of trypanosome marking was obtained when a normal mouse serum was used;

Fig. 15d is a high-magnification (100X) immunocytochemical marking illustrating antibody "C" having an intensity very close to the one for the monoclonal Ab;

Fig. 15e is a high-magnification (100X) immunocytochemical marking illustrating the anti-NO-Tyr ("T") giving a positive marking with an intensity not as high as the two types of antibodies (monoclonal and polyclonal) directed against the epitope NO-Cys;

Fig. 15f is a high-magnification (100X) immunocytochemical marking illustrating the absence of marking in the primary Ab of a normal rabbit;

Fig. 16 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-BSA epitope;

Fig. 17 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on delipidated NO-BSA epitope;

Fig. 18 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Cys-BSA epitope;

Fig. 19 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Cys-G-BSA epitope;

Fig. 20 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tyr-BSA epitope

Fig. 21 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tyr-G-BSA epitope

Fig. 22 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tryp-G-BSA epitope

Fig. 23 illustrates the OD obtained from ELISA tests on the NO-Cys-G-BSA, NO-Tyr-BSA and NO₂-Tyr-BSA conjugates (the control group) and indicating presence of circulating Ab whose rate increases during attacks and decreases during remissions;

Fig. 24 illustrates the OD obtained from ELISA tests on anti-NO-Tyr-BSA and anti-NO₂-Tyr-BSA conjugates (the aminoguanidine group);

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Fig. 25 illustrates the OD obtained from ELISA tests on anti-NO-Cys-G, anti-NO-Tyr and anti-NO₂-Tyr and showing the changes over time in the anti-NO-Cys-G, anti-NO-Tyr and anti-NO₂-Tyr responses (the monoclonal antibody group);

Fig. 26 illustrates the progression of antibodies directed against NO-Cys-G-BSA, NO-Tyr-BSA and NO₂-Tyr-BSA conjugates (the control group);

Fig. 27 illustrates the progression of antibodies directed against NO-Cys-G-BSA, NO-Tyr-BSA and NO₂-Tyr-BSA conjugates (the NIS group);

Fig. 28 illustrates the progression of antibodies directed against anti-NO-Tyr-BSA and anti-NO₂-Tyr-BSA conjugates (the aminoguanidine group); and

Fig. 29 illustrates the progression of antibodies directed against anti-NO-Cys-G, anti-NO-Tyr and anti-NO₂-Tyr conjugates (the monoclonal antibody group).

DETAILED DESCRIPTION OF THE INVENTION--

Please replace the paragraph beginning at page 7, line 25, with the following rewritten paragraph:

B 3 Several types of NOS were cloned and classified in two distinct families: NOS termed constitutive (cNOS) or inducible NOS (iNOS)--

On page 11, line 27, please replace “-Reaction with tyrosine:”, with -- Reaction with tyrosine--

Please replace the paragraph beginning at page 23, line 7, with the following rewritten paragraph:

B 4 The coupling relationship is the number of moles of hapten coupled with a mole of protein--

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Please replace the paragraph beginning at page 24, line 7 and extending to page 24, line 11, with the following rewritten paragraph:

(NE) Vol before x PM hap

B5 where X mg is the quantity of hapten used for the coupling; CPM before is the radioactivity before dialysis; CPM after is the radioactivity after dialysis; Vol before is the volume before dialysis; PM hap is the molecular weight of the hapten.

Please replace the paragraph beginning at page 25, line 11, with the following rewritten paragraph:

B6 Synthesis of NO₂-tyrosine-BSA : The synthesis of this conjugate requires 20 mg of the NO₂-Tyr (Sigma) hapten and 20 mg of BSA. The coupling takes place with carbodiimide following the same protocol described above.

Please replace the paragraph beginning at page 26, line 1, with the following rewritten paragraph:

B7 The polyclonal serums were adsorbed on the corresponding non-nitrolysated conjugates: Tyr-BSA/HSA for the "T" rabbit and Cys-BSA/HSA for the "C" rabbit. (Geffard et al., 1984a; Geffard et al., 1985b; Campistron et al., 1986). The adsorption took place in proportions of 5 mg of conjugate per ml of pure serum. The mixture was incubated for 16 hours at 4°C under agitation and the immunoprecipitates were eliminated by centrifugation for 15 minutes at 10000g. The supernatant is enriched in specific Ig, while the pellet contains the rabbit Ig-carrier protein immune complexes.

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Please replace the paragraph beginning at page 26, line 10, with the following rewritten paragraph:

B8
- To one volume of rabbit polyclonal serum an equal volume of a saturated ammonium sulfate solution $(\text{NH}_4)_2\text{SO}_4$ is added. The mixture was incubated for 1 hour at 4°C, and then centrifuged for 15 minutes at 10000g. The cell (containing the Ig precipitates) is taken up in a minimum volume of TPB buffer and then dialyzed for 3 days in a SPB buffer (Na_2HPO_4 , 0.01 M, NaCl 0.15 M). -

Please replace the paragraph beginning at page 30, line 24, with the following rewritten paragraph:

B9
B10
- Decrease in the OD (B) indicates the presence of competition between the conjugated (NE) hapten which is adsorbed on the microtitration plate and the hapten preincubated with the corresponding antiserum. B_0 is the OD corresponding to the response obtained with the antiserum in the absence of the competitor. A dilution of the antiserum (1/20,000) yielding a OD of approximately 1.0 to 492 nm was chosen for adjustment of the value of B_0 ; the B/ B_0 relationship was used to trace the competition curves of figure 5 obtained with the competitors. -

Please replace the paragraph beginning at page 43, line 8, with the following rewritten paragraph:

B10
- Despite the absence of competition with NO-BSA, indirect ELISA tests demonstrated that the monoclonal Ab recognizes the nitrosylated protein. The supernatant (1/5) and ascite fluid

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(1/30,000) respectively yielded OD of 0.36 ± 0.085 and 0.54 ± 0.12 . The results represent the average and standard deviation of three experiments

Please replace the last line on page 50 (line 32), with the following rewritten line:

(NE) -- NO-Cys-BSA and NO-Cys-G-BSA: --

On page 51, line 25, please replace “-Direct cytotoxicity by NO.”, with -- Direct cytotoxicity by NO--

Please replace the paragraph beginning at page 54, line 3, with the following rewritten paragraph:

Recent work has shown the formation of nitrotyrosines at inflammatory sites (Kaur and Halliwell, 1994). To detect the presence of immunological responses to these epitopes in the serums of rats, $\text{NO}_2\text{-Tyr-BSA}$ and the conjugated nitrosotyrorine (NO-Tyr-BSA) were used. NO-Tyr-BSA was used to correct OD obtained on : $\text{NO}_2\text{-Tyr-BSA}$ and NO-Tyr-BSA

Please replace the paragraph beginning at page 61, line 25, with the following rewritten paragraph:

(NE) The serums of the ten rats drawn for 5 weeks were tested on conjugates NO-Cys-G-BSA ; NO-Tyr-BSA ; $\text{NO}_2\text{-Tyr-BSA}$, and on the corresponding non-nitrosylated conjugates. Figures 26 and 27 show the “Control” and “NIS” groups, respectively, with the progression over time of the antibodies induced against two epitopes: $\text{NO}_2\text{-Tyr-BSA}$ and NO-Tyr-BSA . These results represent the average on two tests. The OD obtained in each group are equivalent on all conjugates tested, each point represents the average and the

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standard deviation of the OD obtained with the 5 rats in the same group for the same conjugate.

Please replace the paragraph beginning at page 62, line 12, with the following rewritten paragraph:

-- For "Aminoguanidine": (Figure 28) anti-NO-Tyr is the highest response;

B13
the OD are between 1.5 and 2 (between the 2nd and 5th weeks). The anti-N02-Tyr response is as large; note an increase in signals between the 1st and 2nd weeks. They stabilize until the 3rd week, then increase slightly toward the 4th week.

(NE) 2
Please insert a “-“ before the first letter of the underlined subheading on the following pages/lines: page, 10, line 5; page 13, line 4; and page 13, line 21.

(NE) 3
Please delete the “.” in front of the first letter of the subheading on the following pages/lines and replace each occurrence with a “-“: page 22, line 14; page 22, line 32; page 23, line 7; page 23, line 10; page 24, line 21; page 25, line 11; page 25, line 34; page 26, line 9; page 26, line 15; page 29, line 32; page 30, line 4; page 31, line 5; page 33, line 18; page 33, line 27; page 34, line 8; page 41, line 8; page 41, line 18; page 46, line 14; page 46, line 16; page 46, line 18; page 46, line 27; page 46, line 29; page 46, line 32; page 53, line 12; page 53, line 18; page 53, line 31; page 54, line 3; page 54, line 14; page 54, line 21; page 54, line 26; page 55, line 22; page 55, line 30; page 56, line 3; page 56, line 23; page 56, line 28; page 56, line 30; page 58, line 13; page 58, line 15; page 58, line 19; page 58, line 21; page 59, line 12; page 59, line 14;